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Monitoring chlorinated persistent organic pollutants in adolescents in Flanders (Belgium): Concentrations, trends and dose–effect relationships (FLEHS II)

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ABSTRACT

Background: In 2007, the second cycle of the Flemish human biomonitoring survey started, with a main focus on 14–15 year-old adolescents.
Objectives: The main objectives were generating reference values for exposure markers, determining the pollution pressure in industrial hotspots and establishing dose–effect relationships between exposure to pollutants and hormone levels, sexual development, asthma and allergy, genotoxic and hematological markers.
Methods: Geometric means with 95% confidence intervals (CI) were calculated for a reference population of 200 14–15 year-old adolescents. Stepwise multiple regression analyses with correction for confounders and covariates were performed to establish dose–effect relationships.
Results: Geometric mean concentrations (with 95% CI) of 49.6 (45.7, 53.8), 70.8 (63.6, 78.8) and 8.34 (7.76, 8.97) ng g^{-1} lipid for the sum of PCB 138, 153 and 180, *p,p′*-DDE and HCB were respectively 23%, 26% and 60% lower than those obtained five years earlier. Geometric mean concentrations of 108 (101, 114) and 32.1 (30.1, 34.2) pg CALUX-BEQ g^{-1} lipid were observed for the PCDD/Fs and dioxin-like PCBs, respectively. Multiple dose–effect relationships were observed between POPs and several effect markers, including positive (boys) and negative (girls) associations with data on sexual development and positive associations with asthma, animal allergy and free thyroxine (boys and girls).
Conclusions: Our findings suggest that chlorinated POP concentrations are decreasing over time and that even relatively low concentrations are associated with biological effects.

1. Introduction

As part of the 'Decree on Preventive Health Care as a legal recognition of environmental health', voted by the Flemish government in 2003, the Flemish Environment and Health Study (FLEHS) continued in 2007 with a second cycle. During the pilot study (1999) and the FLEHS I survey (2002–2006), multiple pollutants and biomarkers of effect were measured in different areas in Flanders (Covaci et al., 2002; Koppen et al., 2002, 2009; Schroijen et al., 2008) and interesting relationships between concentration levels and biological effects were observed (Croes et al., 2009; De Coster et al., 2008; Dhooge et al., 2011; Ketelslegers et al., 2008; Maervoet et al., 2007). The main purpose of this new campaign (2007–2011) was to generate new reference values for several biomarkers and to asses the pollution pressure in selected hotspot areas (i.e. geographical areas or population groups with a concern for environmental pollution pressure) (Schoeters et al., 2012). In total, more than 40 biomarkers of exposure (i.e. various pollutants) and 10 effect markers (i.e. hormones, asthma and allergies and sexual development) were measured in an adolescent population (14–15 years of age). Results of the reference group (Flanders, n = 210) were used as control values for the two adolescent biomonitoring campaigns (twice n = 200) in the hotspot areas "Genk-Zuid" (an industrial area comprising a stainless steel plant in the South-East of Flanders) and "region of Menen" (an industrial area around a shredder, near the French border in the South-West of Flanders). Measurements in adolescents reflect

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local exposure (limited changing of residence) and are not influenced by direct occupational exposure. Younger people also reflect more recent exposure to pollutants (compared to adults who show a cumulative exposure for certain persistent organic pollutants or POPs) and they can be recruited more easily in small areas compared to newborns. Possible influences on the POP body burden of the adolescent from lactational exposure as a newborn could be taken into account, since this information was available from questionnaires. Additionally, data on sexual development were obtained from the school physicians. The main objectives of this paper were 1) presenting new reference values of POP concentrations (dioxins and furans PCDD/Fs, dioxin-like polychlorinated biphenyls dl-PCBs, other non-dioxin-like PCBs, *p,p′*-dichlorophenyldichloroethylene *p,p′*-DDE and hexachlorobenzene HCB), measured in the serum of Flemish adolescents, 2) determining the pollution pressure in the two industrial hotspots and 3) establishing dose–effect relationships between exposure to POPs and effect markers (for the whole group of adolescents enrolled in FLEHS II, $n = 606$).

PCBs were first produced commercially in the 1920s and were often used as hydraulic or transformer fluids and plasticizers in paint and in carbonless copying paper. In Belgium, the use of PCBs has been banned since 1985. *p,p′*-DDE is the metabolite of the pesticide DDT, a product that has been used worldwide to control insects in agriculture and insects that carry diseases such as malaria. Although the use of DDT is forbidden in Belgium for more than 35 years, it is still allowed in certain developing countries with malaria. HCB is a persistent chlorinated pesticide that was used in Belgium until 1974 to protect seeds and grains against fungus. PCDD/Fs are generally formed as a by-product during incomplete combustion processes. In the industrialized countries, PCDD/F contamination increased in the 1930–1940s to reach a maximum in the 1960–1970s. Since then, concentrations have declined as a result of legal actions taken to reduce the emissions. All these POPs are lipophilic, persistent substances that have entered the environment and contaminated the food chain and they all have hormone disrupting, immune disrupting and carcinogenic properties (European Chemical Information System ESIS: http://esis.jrc.ec.europa.eu and International Agency for Research on Cancer IARC: http://www.iarc.fr). Although *p,p′*-DDE, HCB and PCBs were banned in the 1970s–1980s, previous studies showed that the concentration levels in the Flemish population (Croes et al., 2012; Schroijen et al., 2008) and in the environment (Baeyens et al., 2007; Covaci et al., 2005; Windal et al., 2009) are still quite high in certain regions (e.g. in the rural areas). Data from the WHO breast milk surveys showed that PCDD/Fs are still found in relatively high concentrations in breast milk from mothers residing in Flanders (Belgium) compared to mothers from other European and non-European countries (Colles et al., 2008; Croes et al., 2013). Therefore, a major objective of this study was to measure the concentrations of these pollutants in the serum of the Flemish adolescents and to relate the data to several biological endpoints. A targeted approach was used in which measurements of thyroid and sex hormones and data on sexual maturation were used to look at endocrine disruption of the exposure to POPs. Comet assay and urinary 8-hydroxy-deoxyguanosine were assessed as a measure for genotoxicity, while the hematologic parameters and data on asthma and allergies were used as markers for immunotoxicity.

2. Materials and methods

2.1. Selection and recruitment of the participants

Adolescents from selected classes in ten schools in Flanders were invited to participate in the study. Of the 1269 invited adolescents, 51.8% replied to the letter and 69.5% of those that replied gave consent ($n = 456$). From May 2008 to May 2009, 210 of these adolescents (participation rate of 46%) were selected for the reference group after stratification for province, sex and educational level. In the hotspots Genk-Zuid and Menen, a similar approach was used to recruit around 200 adolescents. In Genk-Zuid, 197 adolescents were examined between January and November 2010, while in the region of Menen 199 adolescents were enrolled between May 2010 and February 2011. Due to the limited geographical area of recruitment in the selected hotspot areas and the more diverse social and ethnic background, recruitment was done not only in the schools, but also via home visits with technical support of the local community (community-based participatory research). Nevertheless low participation rates of 34% in Genk-Zuid and 22.5% in the region of Menen were obtained. The study protocol in the reference population and the two hotspot areas (Genk-Zuid and the region of Menen) was identical. Details about inclusion criteria, sampling strategy, communication and a non-responder analysis can be found in the supplementary info. Personal characteristics are described in Table S1. At the time of examination, all adolescents were between 13.6 and 17.0 years old. The number of boys was significantly lower in the hotspot Genk-Zuid (45%) compared to the two other regions (57%), while the mean BMI was higher in this hotspot (21 kg m^{-2} in Genk-Zuid compared to 20 kg m^{-2} in the reference group and hotspot Region Menen). Furthermore, 8.6% (reference group), 5.6% (Hotspot Genk-Zuid) and 7.5% (Hotspot Region Menen) of the adolescents were active smokers. The study design was approved by the medical–ethical committee of the University of Antwerp.

2.2. Analysis of exposure and effect markers

The dioxin-like activity of PCDD/Fs and dl-PCBs in the serum was obtained with the CALUX bioassay, a technique that measures the biologic activity of dioxin-like compounds by means of a receptor based activation of luciferase enzyme in the cell, at the Vrije Universiteit Brussel (VUB), Belgium. Sample pre-treatment and measurement were performed according to the method described by Croes et al. (2011) and the results were expressed as Bioanalytical Equivalents or BEQs. The marker PCBs (PCB 138, 153 and 180), PCB 118, *p,p′*-DDE and HCB were quantified in the serum samples with gas chromatography–mass spectrometry (GC–MS) at the University of Antwerp (Belgium), according to the protocols described by Covaci and Schepens (2001) and by Covaci and Voorspoels (2005). The involved laboratories had to fulfill standard quality assurance and quality control (QA/QC). Validation dossiers were required and participation to international ring tests was desired.

Commercial immunoassays were used to determine serum levels of total testosterone (T) (Medgenix, Fleurus, Belgium), luteinizing hormone (LH), sex hormone binding globulin (SHBG) (Orion Diagnostica, Espoo, Finland) and total 17β-estradiol (E2) (Clinical Assay, DiaSorin s.r.l., Saluggia, Italy; adapted protocol with use of double amount of serum). The aromatase index was defined as the ratio of testosterone to estradiol (T/E2). The free fractions of testosterone and estradiol were calculated from the levels of the total testosterone, respectively estradiol, and the SHBG concentration, assuming a fixed albumin concentration of 4.3 g dL^{-1} (Vermeulen et al., 1999). In Eq. (1) the formula for calculation of free testosterone is given.

$$\begin{aligned}&\text{Total testosterone concentration} \\ &\quad = 23.43 \times \text{Free testosterone concentration} \\ &\qquad + \text{steroid bound SHBG concentration} \\ &\qquad \times (\text{With steroid bound SHBG} = \text{total SHBG} - \text{free SHBG})\end{aligned} \tag{1}$$

Free 3,5,3′-triiodothyronine (fT3), free thyroxine (fT4), and thyroid stimulating hormone (TSH) were determined by direct chemoluminescence immunoassay on a Modular E170 (T0470) auto analyzer. FT3 and fT4 assays are labeled antibody methods involving competitive immunoassay; the TSH assay is a two-site sandwich method (Cobas Elecsys Line; Roche Diagnostics, Vilvoorde, Belgium). DNA damage was assessed from whole-blood samples by comet assay (measuring the percentage of DNA migration) (Van Goethem et al., 1997), while urinary 8-hydroxy-deoxyguanosine was measured as a biomarker of the DNA repair response to oxidative stress by a commercial ELISA

kit (Barregard et al., 2013). The hematological markers (percentages of thrombocytes, reticulocytes, erythrocytes, eosinophiles and the hemoglobin concentration) were measured in the whole blood samples by Algemeen Medisch Laboratorium (AML, Antwerp, Belgium), using routine, validated methods.

Data on asthma (doctor diagnosed and asthma ever reported) and allergy (animal allergy, eczema and hay fever; both doctor diagnosed and self-assessment) and age at menarche in girls were obtained via self-assessment questionnaires.

Data on sexual development were provided by the Centre for Guidance of Pupils. The time period between the blood sampling and the health investigation was less than 10 months. In boys genital and pubic hair development were assessed by school physicians, while in girls breast and pubic hair development were scored using the international scoring criteria of Marshall and Tanner, where 1 is used for the start of puberty while at stage 5 the adult stage is reached (Marshall and Tanner, 1969, 1970).

2.3. Statistical data treatment

Geometric means with 95% confidence intervals (after Ln transformation) were calculated using SAS 9.2. To compare the FLEHS II reference values with the hotspot measurements and with the results of the FLEHS I survey (Schroijen et al., 2008), Analysis of Variance (ANOVA) testing was used. Each group was compared to the FLEHS II reference values and a significance level of 5% was used. To define dose–effect relationships, stepwise multiple linear regression analysis with correction for pre-defined confounders and selected significant covariates was done on the whole dataset (SAS 9.2). Selection of confounders and possible covariates was based on experience from previous studies (Den Hond et al., 2002; Dhooge et al., 2011; Staessen et al., 2001; Van Den Heuvel et al., 2002) and a detailed literature search in PubMed. Selected covariates with a p-value below 0.20 in univariate analysis were used in the multiple regression model, but only stayed in the model when significant ($p < 0.05$). Confounders of data on (free) estradiol, (free) testosterone, reaching the adult stage of total and free testosterone (i.e. concentrations >320 ng dL^{-1} and >6 ng dL^{-1}, respectively), and the aromatase index (ratio testosterone/estradiol) were age, active smoking, hour of blood sampling, and body mass index (BMI). The parameters "illness during the last 14 days" and season were added as covariates to the multiple regression models. Confounders of data LH and FSH were age, BMI and active smoking. Confounders of data on SHBG were age, BMI, active smoking, and having not eaten before sampling of the blood, while alcohol consumption was added as a covariate. Confounders of data on sexual development were age, BMI and active smoking. Confounders of data on thyroid hormones were age, BMI, sex and illness during the last 14 days. Confounders of data on asthma and allergy were age, active smoking and family status of asthma or allergy. Sex and passive smoking were added as covariates to the models with asthma as effect marker. Confounders of data on genotoxicity and hematological markers were age, sex, active smoking and (only for hemoglobin) ferritin. The maximum outdoor air temperature was a covariate for the Comet assay (genotoxicity), while alcohol consumption and BMI were added as covariates for the hematological effect parameters. The limit of quantification (LOQ) was 0.05 pg BEQ g^{-1} serum for the PCDD/Fs and 0.025 pg BEQ g^{-1} serum for the dl-PCBs. The LOQ for PCB 118, HCB, *p,p′*-DDE and marker PCBs yielded 20 ng L^{-1} serum. For samples with POP concentrations below the LOQ, half of the LOQ was used for calculations.

3. Results and discussion

3.1. POP concentrations in the serum of Flemish adolescents

In Table 1, an overview of the geometric mean (GM) POP concentrations (raw, not corrected data) in human serum samples of adolescents (14–15 years old), residing in Flanders (FLEHS I and II) and in specific hotspots (FLEHS II), is given. All POPs could be measured in more than 90% of the serum samples. PCDD/Fs and dl-PCBs were only measured during the FLEHS II campaign.

In the reference group of this study (FLEHS II, $n = 210$), geometric mean concentrations (with 95% CI) of 49.6 (45.7, 53.8), 70.8 (63.6, 78.8) and 8.34 (7.76, 8.97) ng g^{-1} lipid were found for sum of marker PCBs, *p,p′*-DDE and HCB. Geometric mean concentrations of 108 (101, 114) and 32.1 (30.1, 34.2) pg CALUX-BEQ g^{-1} lipid were observed for the PCDD/Fs and dioxin-like PCBs, respectively. In both hotspots (Genk-Zuid, $n = 197$ and region of Menen, $n = 199$) all POPs, except HCB, were significantly lower ($p < 0.05$) compared to the reference mean (multiple ANOVA tests, each comparing data of the reference group and one of the hotspots).

Factors possibly affecting the observed variation in POP values could be, for example, consumption of local food (e.g. eggs) and fat-rich food in general (e.g. meat, milk, eggs), time trends (measurements in Genk and the region of Menen were done two years later compared to the general, reference population) and socioeconomic status (defined as the highest educational level in the family). A detailed analysis concerning factors influencing the observed differences in exposure to POPs is described by Colles et al. (in preparation).

The reference values measured in Flanders in this study (FLEHS II, 2008–2009, $n = 210$) were lower compared to the concentrations levels found five years earlier (FLEHS I, 2003–2004, $n = 1679$). The geometric means for the sum of PCBs, *p,p′*-DDE and HCB were respectively 23%, 26% and 60% lower ($p < 0.001$) in this study than in FLEHS I (Schroijen et al., 2008). Although participants were recruited from the same geographical area during both studies, one has to take into account that during FLEHS I the focus was on eight selected areas in Flanders, while in FLEHS II the selection was more randomized, and that this could have possibly affected the measured mean concentration levels.

The dl-PCBs and PCDD/Fs were only measured for the first time in adolescents in Flanders and could thus not be compared with other Flemish data. Results from human breast milk samples did however show that concentrations of dl-PCBs and PCDD/Fs were also decreasing over time in Flanders. Between 2006 and 2010 the PCDD/Fs decreased with 18% (form 10.3 to 8.4 pg TEQ g^{-1} lipid), while for the dl-PCBs a decrease of 16% was observed (from 7.0 to 5.9 pg TEQ g^{-1} lipid) (Croes et al., 2012, 2013).

Overall, these data suggest that the body burden of POPs is decreasing over time (when comparing FLEHS I and II reference data) and that there are significant differences in concentrations according to area of residence (comparing reference values with the "industrial" hotspot measurements).

3.2. Dose–effect relationships

In this section, all statistically significant relationships between POP concentrations in Flanders and biomarkers of effect (taking into account all data from the FLEHS II reference population and the two hotspots, $n = 606$) are described.

3.2.1. Sex hormones in boys

The sex hormones (total and free testosterone, reaching the adult stage of total and free testosterone, total and free estradiol, the aromatase index, SHBG, LH and FSH) were only available for the boys participating in the study ($n = 324$). This was done because in boys normal values for sex hormone levels during puberty are available (Kletter et al., 1993), while in girls, due to variance in hormone concentration during the menstrual cycle, a similar interpretation is not feasible.

The concentrations of the sum of marker PCBs were positively correlated with SHBG ($p = 0.008$, after Ln transformation), total testosterone ($p = 0.03$) and the aromatase index ($p = 0.001$), while a negative correlation with total and free estradiol was reported ($p = 0.004$ and $p = 0.01$ respectively; both after Ln transformation) (Table 2).

Table 1
Geometric mean (GM) POP levels in human serum: overview of the reference values for Flanders and comparison with two hotspot regions (raw data). Sum PCBs, *p,p*′-DDE and HCB are expressed in ng g^{-1} lipid, PCDD/Fs and dl-PCBs are expressed in pg CALUX-BEQ g^{-1} lipid. Sum PCBs = sum of PCB 138, PCB 153 and PCB 180. na = not analyzed. CI = confidence interval. Significant results ($p < 0.05$) are indicated in bold (ANOVA-test).

Pollutants	Reference values Flanders '03–'04 (FLEHS I) GM (95% CI)	Reference values Flanders '08–'09 (FLEHS II) GM (95% CI)	Hotspot Genk-Zuid '10–'11 GM (95% CI)	% difference with Flanders '08–'09	p-Value	Hotspot Menen '10–'11 GM (95% CI)	% difference with Flanders '08–'09	p-Value
Sum PCBs (ng g^{-1} lipid)	68 (66,70)	49.6 (45.7, 53.8)	31.0 (28. 6, 33.6)	−38%	<**0.001**	37.2 (34.1, 40.6)	−25%	<**0.001**
p,p′-DDE (ng g^{-1} lipid)	94 (89, 99)	70.8 (63.6, 78.8)	47.6 (42.7, 53.1)	−32%	<**0.001**	47.9 (43.2, 53.0)	−32%	<**0.001**
HCB (ng g^{-1} lipid)	21 (20, 21)	8.34 (7.76, 8.97)	7.73 (7.14, 8.37)	−7%	0.17	7.82 (7.25, 8.44)	−6%	0.23
PCDD/Fs (pg BEQ g^{-1} lipid)	na	108 (101, 114)	48.1 (43.7, 53.0)	−55%	<**0.001**	70.0 (65.5, 74.9)	−35%	<**0.001**
dl-PCBs (pg BEQ g^{-1} lipid)	na	32.1 (30.1, 34.2)	10.9 (10.1, 11.8)	−66%	<**0.001**	29.1 (27.5, 30.8)	−9%	**0.03**

This first observation is in line with data from FLEHS I (2002–2006), where also significant positive associations with total and free testosterone and the aromatase index were found (Dhooge et al., 2011). In literature, only two studies were found describing the dose–effect relationship between marker PCBs (in cord blood) and testosterone levels in adolescents: Mol et al. (2002) reported a non-significant trend for higher testosterone levels in boys at the age of 14 with increasing PCB concentrations, while Grandjean et al. (2012) observed a significant negative relationship between marker PCBs in cord blood and free testosterone levels at the age of 14. Unfortunately, these studies relate PCB measurements in cord blood to current hormone levels and the conclusions can thus not be easily extrapolated to our study in which PCBs and hormone concentrations were measured during adolescence. A study from Haugen et al. (2011) did not observe any significant correlations between sex hormones (testosterone, estradiol, LH and FSH) and PCB 153 in young adult men (mean age 26 years). Several other studies reported positive correlations between PCBs and SHBG, which is in agreement with our findings, although in only one study all biomarkers were measured during adolescence. Grandjean et al. (2012) reported a significant positive association between current SHBG levels in boys and marker PCBs (both prenatal PCB concentration in cord blood and current concentration at the age of 14 years old). Two other studies on adult males reported positive correlations between PCB 153 and SHBG and LH concentrations in the blood (Bonde et al., 2008; Giwercman et al., 2006).

In the FLEHS I study, Dhooge et al. (2011) reported, contrary to this study, a positive association for total and free estradiol and the sum of the marker PCB concentration in the blood of the boys. In literature, no studies were found concerning the relationship between estradiol levels and PCB concentrations in children, but negative correlations were observed in other age groups. Cao et al. (2008) observed a negative correlation between marker PCB concentrations in the cord blood of neonates and the levels of estradiol and testosterone. Also studies from Plíšková et al. (2005) and Bonefeld-Jorgensen (2010) showed similar effects on adult males. These negatively associated effects on the estradiol concentration are indeed more expected, since the higher chlorinated PCBs (like PCB 138, 153 and 180) are known to be anti-estrogenic (Krüger et al., 2008). The anti-estrogenic activity of PCBs can also explain the higher concentrations of testosterone (observed in our study), since endogenous estrogens inhibit the testosterone production through actions on the testis or indirectly on the hypothalamic–pituitary axis (Delbès et al., 2005; Sanford, 1985). Handelsman (2008) reported that estrogen blockers can cause a sustained increase in testosterone levels in men.

The dioxin-like PCB 118 was only significantly positive correlated with LH ($p = 0.048$) and FSH ($p = 0.03$) in the blood of boys, while for the dl-PCBs and PCDD/Fs (measured with the H1L7.5c1 mouse CALUX bioassay), no significant relationships were found (Table 2). Data on dose–effect relationships of dioxin-like compounds in adolescents are very scarce. Results of the neonate study in FLEHS II (unpublished results) were in agreement with the adolescent data, showing a positive association between PCB 118 and LH. Literature data are however not always consistent: some studies found negative correlations between dioxin-like compounds (PCBs and PCDD/Fs) in cord blood and testosterone (Cao et al., 2008; Johnson et al., 2001) or estradiol (Cao et al., 2008; Mocarelli et al., 2008), while others reported positive relationships with estradiol (Yang et al., 2005; Yoshida et al., 2005) and FSH (Mocarelli et al., 2008; Yang et al., 2005) concentrations in

Table 2
Dose–effect relationships between chlorinated POP concentrations (exposure) and sex hormones (effect, all Ln-transformed), measured in boys (n = 324).

Exposure	Effect	Confounders	Covariates	Estimate[a] (95% CI)	IQR	p-Value
Sum marker PCBs (ng g^{-1} lipid)	Estradiol (pg mL^{-1})	Age, blood collection before 11 h, BMI, active smoking	–	0.90 (0.85;0.97)	31.4	0.004[b]
Sum marker PCBs (ng g^{-1} lipid)	Free estradiol (pg mL^{-1})	Age, blood collection before 11 h, BMI, active smoking	Season, illness last 14 days	0.86 (0.76;0.96)	31.4	0.01[b]
HCB (ng g^{-1} lipid)	Testosterone (ng dL^{-1})	Age, blood collection before 11 h, BMI, active smoking	–	1.04 (1.01;1.07)	4.84	0.004
Sum marker PCBs (ng g^{-1} lipid)				1.03 (1.00;1.06)	31.4	0.03
HCB (ng g^{-1} lipid)	Aromatase index	Age, blood collection before 11 h, BMI, active smoking	–	1.05 (1.01;1.08)	4.84	0.007
Sum marker PCBs (ng g^{-1} lipid)				1.06 (1.02;1.10)	31.4	0.001
Sum marker PCBs (ng g^{-1} lipid)	SHBG (nmol L^{-1})	Age, BMI, active smoking, having not eaten	Alcohol consumption	1.08 (1.02;1.15)	31.4	0.008[b]
PCB 118 (ng g^{-1} lipid)	LH (mU mL^{-1})	Age, BMI, active smoking	–	1.04 (1.00;1.07)	1.84	0.048
PCB 118 (ng g^{-1} lipid)	FSH (mU mL^{-1})	Age, BMI, active smoking	–	1.05 (1.01;1.09)	1.84	0.03

Exposure	Effect	Confounders	Covariates	Odds ratio[c] (95% CI)	IQR	p-Value
HCB (ng g^{-1} lipid)	Reaching adult stage of testosterone (%)	Age, blood collection before 11 h, BMI, smoking	–	1.29 (1.01;1.65)	4.84	0.04

[a] Interpretation estimate (regression coefficient) for continuous markers of exposure: if the exposure increases with the interquartile range (IQR), the mean effect is multiplied with the estimate.
[b] p-Value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on a Ln-transformed scale.
[c] Interpretation odd ratio (OR): if the exposure increases with the IQR, the odds for the effect marker are multiplied with the factor OR.

the cord blood. Mocarelli et al. (2011) showed that exposure to dioxins resulted in reduced sperm quality, increased FSH and decreased inhibin B levels in young adults. Higher concentrations of FSH in relationship with dioxin-like compounds are probably due to a dysfunction of the Sertoli cells. It is known that dl-PCBs and other dioxin-like compounds act through the AhR–ARNT complex that regulates or directly intervenes in reproductive system development (Grandjean et al., 2012; Mocarelli et al., 2011).

Hexachlorobenzene (HCB) was positively correlated with total testosterone ($p = 0.004$), reaching the adult stage of testosterone ($p = 0.04$, $OR = 1.29$) and the aromatase index ($p = 0.007$) (Table 2). A positive association between HCB and testosterone levels and the aromatase index was also found for the boys in the FLEHS I study (Dhooge et al., 2011). The pesticide metabolite *p,p′*-DDE was not significantly correlated with sex hormone levels in the boys in our study.

3.2.2. Degree of sexual maturation in boys and girls

Data on sexual maturation was obtained for both boys (pubic hair and genital development) and girls (pubic hair and breast development and age at reaching menarche) (Table 3).

For the girls in this study a negative relationship between marker PCB concentrations and breast and pubic hair development (percentage of girls reaching stage of 4 or 5 on the scale of Marshall and Tanner) was found ($p = 0.02$, $OR = 0.52$ and $p = 0.02$, $OR = 0.49$ respectively), supporting the hypothesis of anti-estrogenic effects of PCBs and also in agreement with the negative relationship between estradiol levels and PCB concentrations in boys. In a study on 9-year-old girls from New York also a negative correlation between breast development and PCB concentration in the blood was found (Wolff et al., 2008). This is again an indication of the anti-estrogenic effect of the marker PCBs. It can thus be concluded that the positive associations of marker PCBs with (free) testosterone and the negative relations with (free) estradiol in boys, and the negative associations with sexual maturation in girls are due to the anti-estrogenic activity of PCBs.

The total amount of dl-PCB, measured with the CALUX bioassay, was negatively correlated with breast development in girls ($p = 0.04$, $OR = 0.56$ after Ln transformation) (Table 3). In a study in Flanders (1999), also a negative correlation was found between breast development in 14–15 year-old girls and the sum of dl-PCBs and PCDD/Fs (Den Hond et al., 2002), while in a Dutch study breast development was negatively associated with prenatal and lactational concentrations of PCDD/Fs (Leijs et al., 2008). Our results show thus that there seems to be a negative correlation between exposure to dioxin-like compounds and breast development in girls (Staessen et al., 2001 survey from 1999 and current study).

For girls, also a negative association between HCB and reaching menarche at the age of 14–15 years old ($p = 0.02$, $OR = 0.35$) was reported (Table 3). In a study in Canada (girls aged 10–17 years), a negative association was found between age at menarche and the sum of 4 estrogenic PCBs (PCB 52, 70, 101, 187), but no relationship was found with HCB and *p,p′*-DDE (Schell and Gallo, 2010). No other literature data were available. The pesticide metabolite *p,p′*-DDE was only negatively correlated with breast development in girls ($p = 0.03$, $OR = 0.74$). For the boys in the study, only a borderline significant positive correlation between pubic hair development and HCB was observed ($p = 0.052$, $OR = 1.77$) (Table 3).

3.2.3. Thyroid hormones

Dose–effect relationships on thyroid hormones (fT4, fT3 and TSH) were analyzed for all adolescents ($n = 606$).

All chlorinated POPs (marker PCBs, *p,p′*-DDE, HCB and dl-PCBs and PCDD/Fs (measured with the CALUX bioassay)) were positively correlated with fT4 (p values of respectively 0.0004, 0.02, 0.002 after Ln-transformation, 0.03 after Ln-transformation and 0.004). TSH was positively associated with HCB ($p = 0.02$), while a negative correlation with PCDD/Fs ($p = 0.02$ after Ln transformation) was observed. No significant relationships with fT3 were found (Table 4).

A significant positive relationship between fT4 and PCBs was also found in the FLEHS I study (unpublished results). However, in most animal studies and in a large part of the published human surveys negative associations between total and free T4 and chlorinated POPs are described (Arisawa et al., 2005; Boas et al., 2006; Hagmar, 2003 and references herein). On the other hand, several studies on adults reported negative correlations between PCBs and (f)T4: 1) Persky et al. (2001) and Turyk et al. (2006): found negative associations between the sum of 89 PCBs and T4 (adult men, residing in the USA); 2) Persky et al. (2001) also reported the same finding for both T4 and fT4 and the sum of 89 PCBs in adult women from the USA; 3) Abdelouahab et al. (2008) found negative associations between the sum 16 PCBs and T4 in Canadian adult men; and 4) Alvarez-Pedrerol et al. (2008), observed negative associations between PCB 118 and fT4 in 4-year-old Spanish children. A survey on Canadian adolescents (10–17 years old) also found a negative association between fT4 and the sum of all and the individual PCB congeners PCB 138, 153, 52, 70, 84, 87, 101 and 149 (Schell et al., 2008). Bloom et al. (2003), Rylander et al. (2006) and Hagmar et al. (2001) could not find any significant association between (f)T4 and the sum of PCBs. Only one study reported a positive association with the sum of 15 PCB congeners (PCB 28, 52, 101, 105, 114, 118, 123, 138 + 163, 153, 156 + 171, 157, 167, 170, 180 and 189), but this relationship was only valid for PCB concentrations higher than 1000 ng g^{-1} lipids. For PCB concentration in the lowest quintile (lower than 530 ng g^{-1} lipids), also a negative association was observed (Langer et al., 2007).

Turyk et al. (2007) reported an inverse association between total T4 and the total TEQ of PCDD/Fs and dl-PCBs in the blood of adult men and women, while a positive association between total T4 and *p,p′*-DDE in the blood of women was found. A positive association between *p,p′*-DDE and total T3 and fT4 was also found by Meeker et al. (2007) in adult men, while Schell et al. (2008) reported a negative correlation between total T4 and HCB in a study on Canadian adolescents, aged between 10 and 17 years old.

It is evident that thyroid function shows a complex relation with endocrine disrupting substances. Disruption of thyroid function happens to a large extent at the level of pre-receptor regulation of ligand availability (Crofton et al., 2005; Köhrle, 2008). Better understanding of

Table 3
Dose–effect relationships between chlorinated POP concentrations (continuous exposure marker) and sexual development (binary effect marker), measured in boys ($n = 324$) and girls ($n = 282$).

Exposure	Effect	Confounders	Covariates	Odds ratio[a] (95% CI)	IQR	p-Value
HCB (ng g^{-1} lipid)	Pubic hair development boys	Age, BMI, active smoking	–	1.77 (1.00;3.14)	4.84	0.052
HCB (ng g^{-1} lipid)	Reaching menarche (%)	Age, BMI, active smoking	–	0.35 (0.15;0.84)	4.84	0.02
p,p′-DDE (ng g^{-1} lipid)	Breast development girls	Age, BMI, active smoking	–	0.74 (0.57;0.98)	53.3	0.03
Sum marker PCBs (ng g^{-1} lipid)				0.52 (0.30;0.92)	31.4	0.02
dl-PCBs (pg BEQ g^{-1} lipid)				0.56 (0.32,0.97)	21.6	0.04[b]
Sum marker PCBs (ng g^{-1} lipid)	Pubic hair development girls	Age, BMI, active smoking	–	0.49 (0.27;0.87)	31.4	0.02

[a] Interpretation odd ratio (OR): if the exposure increases with the interquartile range (IQR), the odds for the effect marker are multiplied with the factor OR.
[b] p-Value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on a Ln-transformed scale.

Table 4
Dose–effect relationships between chlorinated POP concentrations (exposure) and thyroid hormones (effect, Ln-transformed), measured in the whole group (n = 606).

Exposure	Effect	Confounders	Covariates	Estimate[a] (95% CI)	IQR	p-Value
HCB (ng g^{-1} lipid)	TSH (μU mL^{-1})	Age, BMI, sex, illness last 14 days	–	1.03 (1.01;1.05)	4.84	0.02
PCDD/F (pg BEQ g^{-1} lipid)				0.94 (0.89,0.99)	67.9	0.02[b]
HCB (ng g^{-1} lipid)	Free T4 (ng dL^{-1})	Age, BMI, sex, illness last 14 days	–	1.02, (1.01;1.03)	4.84	0.002[b]
p,p′-DDE (ng g^{-1} lipid)				1.003 (1.001;1.006)	53.3	0.02
Sum marker PCBs (ng g^{-1} lipid)				1.01 (1.01;1.02)	31.4	0.0004
dl-PCBs (pg BEQ g^{-1} lipid)				1.01 (1.00,1.03)	21.6	0.03[b]
PCDD/F (pg BEQ g^{-1} lipid)				1.01 (1.00;1.02)	67.9	0.004

[a] Interpretation estimate (regression coefficient) for continuous markers of exposure: if the exposure increases with the interquartile range (IQR), the mean effect is multiplied with the estimate.
[b] p-Value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on a Ln-transformed scale.

this complex relation may require additional large scale studies in which not only (f)T4, (f)T3 and TSH, but also thyroxine-binding globulin (TBG), transthyretin (TTR, or prealbumin) and albumin are measured in conjunction with determination of the concentration of the many chemical substances that can contribute to disruption of the thyroid gland function, including phenols, phthalates, UV-filters (Hofmann et al., 2009), brominated flame retardants (Kuriyama et al., 2007; Schreiber et al., 2010) and perfluorinated compounds (Melzer et al., 2010).

3.2.4. Asthma and allergy

The POP marker PCBs (p = 0.007, OR = 1.58 after Ln transformation) and *p,p′*-DDE (p = 0.006, OR = 1.61 after Ln transformation) were positively associated with doctor-diagnosed asthma (Table 5).

Sunyer et al. (2005) also reported a positive association between *p,p′*-DDE concentrations in cord blood and diagnoses of asthma at the age of 4 years, while Karmaus et al. (2001) came to the same conclusion in a survey on German children (OR = 3.71). In a Flemish study (1999) on 17–18 year old adolescents, a positive relationship between marker PCBs and having ever had asthma was found (Van Den Heuvel et al., 2002). Grandjean et al. (2010) found a weak correlation between prenatal marker PCB concentrations and development of asthma at the age of 7 years. Although very few studies on children are present, it seems that chlorinated POPs can increase the prevalence of asthma and that this assumption is valid for both young and older children.

The dl-PCBs were positively associated with the development of animal allergy (p = 0.02, OR = 1.46), while a borderline non-significant correlation was found between dl-PCBs and development of hay fever (p = 0.11, OR = 1.23 after correction for age, active smoking and familial history of hay fever) and eczema (p = 0.10, OR = 0.73, after correction for age, active smoking and familial history of eczema). Higher PCDD/F levels were associated a higher risk for development of animal allergy (p = 0.03, OR = 1.60 after Ln transformation) and asthma (p = 0004, OR = 1.42) (Table 5). Only few studies on children concerning the relation between dioxin-like compounds and allergies were found. A Dutch study on 8-year-old children reported a significant lower risk for development of allergies (hay fever and animal allergy analyzed together) with higher pre- and postnatal PCDD/F concentrations (Ten Tusscher et al., 2003). In a Norwegian study a negative correlation was found between prenatal marker PCBs, dl-PCBs and PCDD/Fs and the development of eczema (Stølevik et al., 2011). In both studies, the pollutants were measured in pre- or postnatal blood samples, while in our survey pollutant concentrations and effects were obtained from 14–15 year old adolescents. This could explain the different observations, but more research is necessary since the interaction of exposure to POPs and immunologic function is very complex and incompletely understood.

It is known that TCDD may aggravate allergic diseases by enhancing IgE-mediated allergic responses (Kimata, 2003). However, a recent systematic review of 41 studies by Gascon et al. (2013) found limited evidence for prenatal exposure to *p,p′*-DDE, PCBs and dioxins and risk of respiratory infections. Evidence was limited also for postnatal exposure to PCBs, specifically non-dl-PCBs, and reduced immune response after vaccination in childhood. The review indicates lack of association between postnatal exposure to PCBs and risk of asthma-related symptoms. For the other exposure–outcome associations reviewed evidence was inadequate.

3.2.5. Genotoxic and hematologic effect markers

No significant correlations were found between chlorinated POPs and genotoxic parameters. The percentage thrombocytes in the blood was negatively associated with the marker PCBs (p = 0.04 after Ln transformation), dl-PCBs (p = 0.02) and PCDD/Fs (p = 0.0005), while the hemoglobin concentration was positively associated with the marker PCBs (p = 0.046) and with PCB 118 (p = 0.02) (Table 6). No significant correlations were found between the POPs and reticulocyte, erythrocyte or eosinophile concentrations in the blood of the participants.

In literature, data concerning genotoxic and hematologic effects are very scarce. Only two Dutch studies describe the relationship between blood parameters and dioxin-like compounds in the blood of children. In a group of children, aged 8 years, a significant negative association was found between the concentration of PCDD/Fs and thrombocytes (Ten Tusscher et al., 2003). This finding was also reported within the same individuals during their neonatal period (Pluim et al., 1994) and seemed thus to be persistent. In a study on 14–19 year old children no significant relationships between dl-PCBs and PCDD/Fs and thrombocytes and hemoglobin were observed (Leijs et al., 2009). In the study of Ten Tusscher et al. (2003) the lower number of thrombocytes was inverse related to the concentration of thrombopoietin, a protein made in the liver that regulates the number of thrombocytes. The authors

Table 5
Dose–effect relationships between chlorinated POP concentrations (continuous exposure marker) and asthma and allergy (binary effect marker), measured in the whole group (n = 606).

Exposure	Effect	Confounders	Covariates	Odds ratio[a] (95% CI)	IQR	p-Value
p,p′-DDE (ng g^{-1} lipid)	Asthma doctor diagnosed	Age, active smoking, asthma in family	–	1.61 (1.14; 2.27)	53.3	0.006[b]
Sum marker PCBs (ng g^{-1} lipid)				1.58 (1.13; 2.19)	31.4	0.007[b]
PCDD/F (pg BEQ g^{-1} lipid)	Asthma in the last 12 months	Age, active smoking, asthma in family	Passive smoking	1.42 (1.17;1.72)	67.9	0.0004
dl-PCB (pg BEQ g^{-1} lipid)	Animal allergy	Age, active smoking, animal allergy in family	–	1.46 (1.07;1.98)	21.6	0.02
PCDD/F (pg BEQ g^{-1} lipid)				1.60 (1.04,2.67)	67.9	0.03[b]

[a] Interpretation odd ratio (OR): if the exposure increases with the interquartile range (IQR), the odds for the effect marker are multiplied with the factor OR.
[b] p-Value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on a Ln-transformed scale.

Table 6
Dose–effect relationships between chlorinated POP concentrations (exposure) and genotoxic and immunologic effect markers (Ln-transformed) measured in boys and girls from the reference group (n = 210).

Exposure	Effect	Confounders	Covariates	Estimate[a] (95% CI)	IQR	p-Value
Sum marker PCBs (ng g^{-1} lipid)	Thrombocytes (%)	Age, sex, active smoking	–	0.96 (0.93;1.0)	31.4	0.04[b]
PCDD/Fs (pg BEQ g^{-1} lipid)			Alcohol consumption	0.91 (0.87;0.96)	67.9	0.0005
dl-PCBs (pg BEQ g^{-1} lipid)				0.94 (0.90;0.99)	21.6	0.02
Sum marker PCBs (ng g^{-1} lipid)	Hemoglobin (g dL^{-1})	Age, sex, ferritin, active smoking	BMI	1.003 (1.000;1.006)	31.4	0.046
PCB 118 (ng g^{-1} lipid)				1.004 (1.001;1.006)	1.84	0.02

[a] Interpretation estimate (regression coefficient) for continuous markers of exposure: if the exposure increases with the interquartile range (IQR), the mean effect is multiplied with the estimate.
[b] p-Value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on a Ln-transformed scale.

hypothesized that the lower number of thrombocytes was thus due to a suppressed production and not to an increase in destruction. Unfortunately, thrombopoietin was not measured in our study, so these finding could not be confirmed.

4. Strengths and weaknesses

A major strength of this cross sectional study is that at the same time a whole range of biomarkers of exposure and effect were measured for a high number of participants. Moreover, information on several potential covariates (e.g. education level, breastfeeding history, food consumption, passive smoking) was available and taken into account in the analyses.

An important limitation is that only information of the internal doses of the pollutants at the age of 14–15 and not at other age windows, which may be sensitive as well to explain the observed dose–effect relationships, is available. Furthermore, information on additional effect markers that were not measured in this study would aid to explore possible mechanisms for some observed relationships. For example, the associations between POPs and thyroid hormones, observed in this study and in FLEHS I, could not be explained with measurements of only three thyroid hormones.

5. Conclusions

Reference values for the sum of PCBs, *p,p′*-DDE and HCB were respectively 23%, 26% and 60% lower in this study than in FLEHS I. The dl-PCBs and PCDD/Fs were only measured for the first time in adolescents' serum in Flanders, but measurements in breast milk samples (Croes et al., 2013) indicated the same declining trend for these POPs. Sex hormones and sexual development in boys were positively associated with chlorinated POPs, except for (free) estradiol, where a negative association was found (only significant for marker PCBs). This can be explained, since the higher chlorinated PCBs are known to be anti-estrogenic. Breast and pubic hair development and reaching menarche in girls were negatively associated with the POP levels in blood. These opposite findings between boys and girls are not surprising, since previous research showed that multiple associations between organochlorine pollutants and gene expression results are gender specific (De Coster et al., 2013).

Chlorinated POPs were positively associated with fT4 levels, which is quite remarkable, since in literature often significant negative associations are found. Furthermore, chlorinated POPs were positively correlated with having asthma, a finding that was also reported by other researchers (Grandjean et al., 2010; Karmaus et al., 2001; Van Den Heuvel et al., 2002). Several dose–effect relationships were also reported for allergies and hematological markers, but more research will be necessary to interpret these results.

Overall it can be concluded that the POP concentration levels are decreasing over time. This is probably due to policy actions following the legislation on the disposal and emission of these POPs. However, although the use and production of PCBs, DDT and HCB were banned in Europe more than 25 years ago, these compounds can still be detected in the serum of most of the adolescents participating in our study. Furthermore, dose–effect relationships show that even the relatively low concentrations found in the blood of the adolescents can have significant effects. Follow-up of the concentrations levels of these historical pollutants in the human body is thus still advisable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2014.05.022.

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